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NON-VARIABILITY OF DIPHTHERIA BACILLI.*

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In 1908 Goodman[1] reported a series of tests with diphtheria bacilli by which he claimed to have established the fact of a natural process of selection among diphtheria organisms, and stated that by taking advantage of this he had been able to obtain, from a strain showing medium acid production in glucose broth, a diverging series, leading to two opposite extremes with regard to acid production.

Starting with a single colony from a case of clinical diphtheria he inoculated a series of tubes of sugar-free infusion broth containing 1 per cent dextrose, incubated for three days at 37° C., and then titrated to determine the final acidity. From the cultures showing the highest and lowest acidity 15 tubes of glucose broth each were inoculated, incubated, and titrated as before. In this manner the two strains, called the High and the Low respectively, were now carried through 36 transfers, the culture showing the maximum acidity being the only one selected each time to carry on in the High Series, and the one with the minimum acidity in the Low Series, plating weekly to avoid contamination.

While at first some irregularity was noticed, Goodman states that he soon found a gradually increasing variation in fermenting power between his High and his Low strains, until at the end of his experiment he had as wide a divergence as between a diphtheria and a pseudo-diphtheria strain, leading on the one hand to a very high acid-producing organism, and on the other to an organism producing alkali in glucose broth.

On the basis of this work he suggests that the division into several distinct species is probably based on a misconception, and that all diphtheria-like organisms are probably only variants of the one species.

Winslow and Walker,[2] taking up the same problem, consider that an important factor in Goodman's work was probably the

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[1] *Jour. Infect. Dis.*, 1908, 5, p. 184.

[2] *Ibid.*, 1909, 6, p. 90.

progressive effect of environment, the organisms being exposed for transfer after transfer to high and low acidities respectively, and therefore likely to gain or lose tolerance to the acid reaction. They planned by excluding this factor to deal only with spontaneous variations. Selecting strains of the two types A and B of the paratyphoid bacillus of Schottmüller, they plated and fished 100 colonies from each into agar tubes.

As soon as these showed distinct growth each was transferred to a tube of 1 per cent glucose broth, grown 72 hours at 20° and titrated. Next, going back to the original agar slants, those fishings which had shown the highest and the lowest acidities in glucose broth were selected to carry on for the High and Low types of each organism, and from each of these, plated out, a series of glucose tubes were inoculated and carried on as before.

The conclusion of this experiment showed quite opposite results to those reported by Goodman with the diphtheria bacillus. With an occasional overlapping in acid production, each series varied around a distinct center, and in each there was a complete reversion to the original mean of the type, no apparent modification of character having been produced by the above process. The entire 538 cultures of the two paratyphoid types fall between the limits of 1.15 and 1.95 acid, yet in this narrow range are evident two distinct modes corresponding to the two apparently distinct paratyphoid types.

Buchanan and Truax,[1] following the method of Goodman, carried out a similar experiment with *Str. lacticus* but failed entirely to obtain fixed high and low acid races of this organism.

Glenn,[2] working with organisms of the proteous type, also found that selection of slight variation did not result in the production of any fixed change as to acid production.

Our work along these lines was carried out with two strains of diphtheria organisms selected from among the 100 diphtheria cultures which had been previously obtained from the throats of diphtheria patients for use in a different study, and which had all been cultivated for many transfers on artificial media. Each of

[1] *Jour. Infect. Dis.*, 1910, 5, p. 680.

[2] *Centralbl. f. Bakt.*, 1911, 6, p. 481.

these 100 strains was transferred to glucose broth, grown for three days in the incubator, and then titrated with phenolphthalein as an indicator, the results being tabulated according to acid production. A second test of the 100 strains was made in the same manner, and two strains were then selected for further experiment. One of these, known as No. 71, while always showing entirely typical organisms on Loeffler blood serum, grew with difficulty in broth when first isolated, producing no pellicle until after long cultivation, and showing dark, coarsely granular, atypical colonies on agar. This organism produced but little toxin in broth, requiring 0.1 c.c. to kill a small guinea-pig in four days, and the preliminary titrations showed but little acid production in glucose broth. The other strain, No. 51, was active in all respects, growing vigorously and typically from the beginning in all media, producing sufficient toxin in broth to kill guinea-pigs in two days with 0.01 c.c., and in six days with 0.005 c.c. and showing a high acid production in glucose broth. It was thought that by starting with these two strains, already at opposite poles of the scale of ordinary variation, there would be the most favorable opportunity offered for the development of Goodman's natural selection process.

The glucose broth used in the experiment was made in the chemical laboratory under Dr. Banzhaf's direct supervision, and the titrations were carried out by Miss Fisher and Miss Stryker under Dr. Banzhaf's direction. The broth was made as follows:

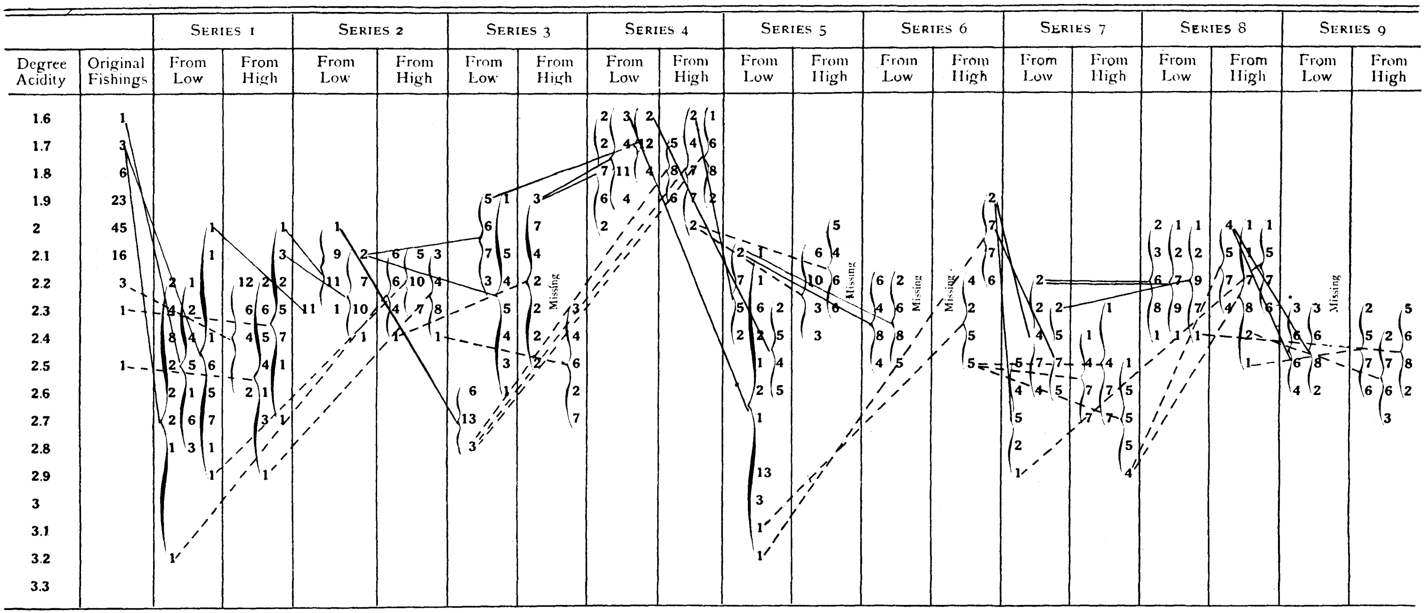
> The clean muscle meat from young veal was ground up. Tap water was added (1 liter water to 1 pound of ground-up meat). An 18-hour colon culture in broth was added. This mixture was allowed to ferment about 20 hours at 22° C. It was then digested for two hours at about 55° C., boiled for 15 minutes, and the meat strained off. Two per cent peptone and 0.5 per cent of salt was added. The medium was then titrated using phenolphthalein as an indicator. Sufficient normal sodium hydroxide was added to adjust the acidity to 1.2 per cent normal acid. Boiled for 15 minutes and filtered clear.
>
> The titrations were carried out in the original tubes of glucose broth culture. Each tube contained 10 c.c. of the medium. Phenolphthalein was used as the indicator. Decinormal sodium hydroxide was used to determine the acidity. The titration was carried on, against a white background, until a faint pink color appeared in the medium. With practice the faint pink color in the medium can easily be distinguished.

The two strains selected for the experiment were plated out on agar, and 100 colonies from each strain were fished on to the surface

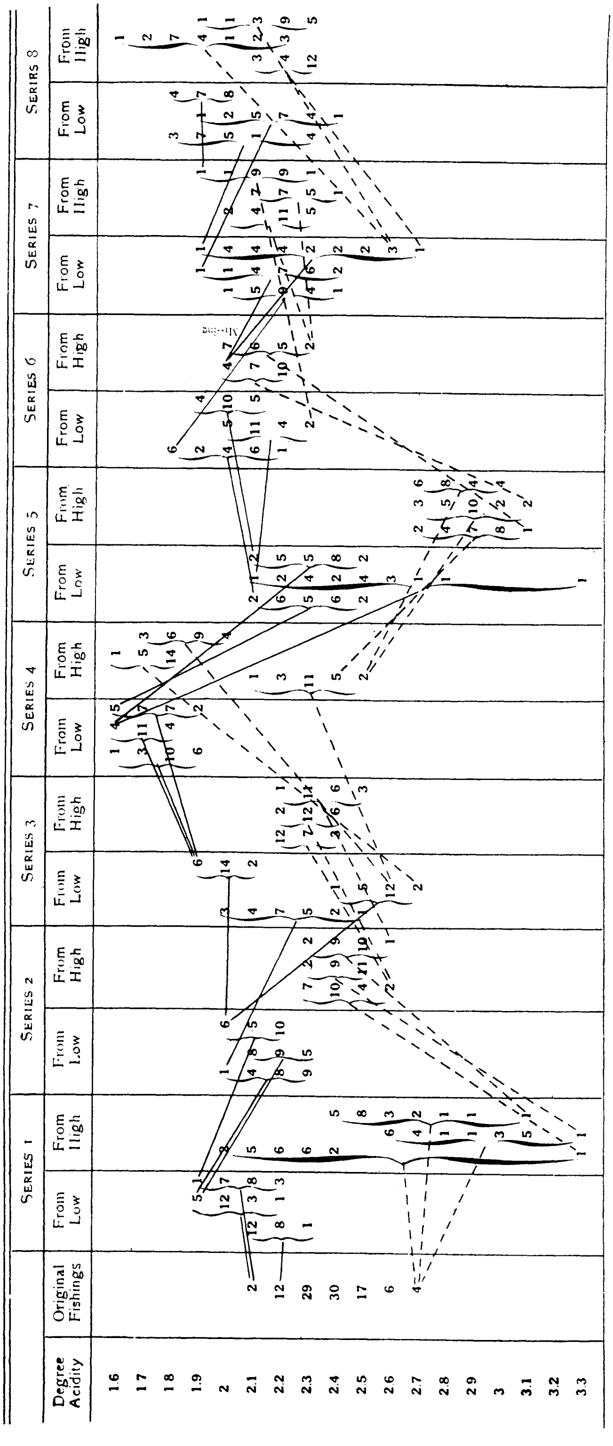
TABLE.

TITRATION OF GLUCOSE BROTH CULTURES.

No. 51.



No. 77.


Degree Acidity
1.6
1.7
1.8
1.9
2
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
3
3.1
3.2
3.3
Original Fishings
2
12
29
30
17
6
4
Series 1
Series 2
Series 3
Series 4
Series 5
Series 6
Series 7
Series 8
From Low
From High

of agar slants over which 0.1 c.c. of ascitic fluid had previously been dropped from a pipette. The fishing needle was washed off in the water of condensation and ascitic fluid in the bottom of the tube, and this was then floated up over the surface of the agar. By this method a good general growth was produced over the surface of the slant, thus avoiding the usual irregular growth from fishings.

The agar cultures, after 24 hours in the incubator, were washed off with 1 c.c. of ascitic fluid into tubes of glucose broth which were grown in the incubator for three days and then titrated as above described.

The result of these titrations showed a different ratio of acid production to that given by the two strains in the preliminary tests, but as the two trials then made had agreed in showing high acidity in No. 51, and a low grade in No. 77, and because of their suitability in other respects, it was decided to go on with the experiment as planned, using these two strains.

Going back to the original agar tubes, which had been returned to the incubator, and again showed a good growth, the three fishings were selected which had shown the highest acid production, and also the three showing the lowest acid production in each strain. These were all plated out, and from 16 to 20 or more colonies of each kind fished into tubes of agar slants plus ascitic fluid, as before, thus averaging about 60 fishings each for the new High and new Low series in each strain. These were now transferred to glucose broth, grown and titrated as before, and again the three highest and three lowest selected for further use.

By proceeding in this way it was hoped to eliminate mere accidental variations, and to give the fullest opportunity for the influence of natural selection. No. 77 was thus carried through eight, and No. 51 through nine series of cultures and titrations, with occasional sets missing owing to breakage or contamination. The results are given in the accompanying table, where the figures show the number of fishings giving the same amount of acid in each set, and the connecting lines show the source of the successive groups.

It will be seen that throughout this experiment there is no evidence whatever of the progressive development described by Goodman. Although there seemed at first some indication of a tendency toward division on the basis of acid production, this soon disappeared so completely that the most striking feature of the whole experiment, with both strains, was the tendency shown by the various fishings, not to diverge, but to approach each other in the degree of acid productions. The irregularity was marked, so much so that conditions were sometimes reversed between two series, the Low of one furnishing the High of the next succeeding series and vice versa, but at the end of the experiment all fishings were seen to be grouped within a very narrow range of acid production in each strain.

It may be said that results might have been different had we carried the experiment farther, but with the large number of substrains used at each transfer, together with the improved technic suggested by Winslow and Walker, it seemed to us that if a tendency to fixed variation exists in the diphtheria bacillus, there should have been at least some indication of it in our series, amounting to over 1,000 titrations for No. 77, and over 1,100 titrations for No. 51.

We cannot exclude the possibility that Dr. Goodman in his experiment may have worked with a mutating organism, but in the extensive work carried out for many years at the Research Laboratory no evidence has ever been found of the existence of such mutation among diphtheria bacilli. The result of the present experiment, moreover, seems to furnish another proof of the fixed characteristics and non-variable nature of pure cultures of the diphtheria organism.

Thanks are due to Dr. Park and Dr. Williams for advice and suggestions in this and the preceding study.